

09/03/99

jc674 U.S. PTO

Please type a plus sign (+) inside this box ☐Approved for use through 9/30/00, OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control numberUTILITY
PATENT APPLICATION
TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 960296.95581

First Inventor or Application Identifier Arnold E. Ruoho

Title BACTERIORHODOPSIN/ G PROTEIN-COUPLED RECEPTOR CHIMERAS

Express Mail Label No. EJ776142175US

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

- 1 ☒ Fee transmittal Form
(Submit an original and a duplicate for fee processing)
- 2 ☒ Specification [Total Pages 51]
(preferred arrangement set forth below)
- Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed Sponsored R&D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
- 3 ☐ Drawing(s) (35 USC 113) [Total Sheets ☐
4. Oath or Declaration [Total Pages ☐
- a. ☒ Newly unexecuted (original or copy)
- b. ☐ Copy from prior Application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
- [Note Box 5 below]
- i. ☐ DELETION OF INVENTOR(S)
Signed Statement attached deleting
inventor(s) named in prior application,
see 37 CFR 1.63(d)(2) and 1.33(b).
- 5 ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application from
which a copy of the oath or declaration is supplied
under Box 4b, is considered as being part of the
disclosure of the accompanying application and is
hereby incorporated by reference herein.
6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
- a. ☒ Computer readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

- 8 ☐ Assignment Papers (cover sheet & documents)
- 9 ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(where there is an assignee)
- 10 ☐ English Translation Document (if applicable)
- 11 ☐ Information Disclosure
Statement (IDS)/PTO-1449 ☐ Copies of IDS
Citations
- 12 ☐ Preliminary Amendment
- 13 ☐ Return receipt postcard (MPEP 503)
(Should be specifically itemized)
- 14 ☐ *Small Entity Statement filed in prior application
Statement(s) ☐ Status still proper and desired
- 15 ☐ Certified copy of priority Document(s)
(if foreign priority is claimed)
- 16 ☐ Other:

* A new statement is required to pay small entity fees, except where
one has been filed in a prior application and is being relied upon

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application no. _____

Prior application information: Examiner: _____ Group/Art Unit: _____

18. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Label

(Insert Customer No. or Attach bar code label)

or ☒ Correspondence address below

NAME	Fahrlander, Jill A.			
	Quarles & Brady			
ADDRESS	P O Box 2113			
CITY	Madison	STATE	WI	ZIP CODE 53701-2113
COUNTRY	US	TELEPHONE	608/251-5000	FAX 608/251-9166

Name (Print/Type)	Jill A. Fahrlander	Registration No. (Attorney/Agent)	42,518
Signature		Date	September 3, 1999

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231.

DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231. QBMA\197346

Firststar Plaza
P.O. Box 2113
Madison, Wisconsin 53701-2113
608/251-5000
FAX 608/251-9166
<http://www.quarles.com>

Attorneys at Law in
Milwaukee and Madison, Wisconsin
West Palm Beach and Naples, Florida
Phoenix, Arizona



September 3, 1999

Assistant Commissioner of Patents
Box Patent Application
Washington DC 20231

Re: Filing New Patent Application

Dear Sir:

Enclosed for filing please find a new patent application entitled:

BACTERIORHODOPSIN/ G PROTEIN-COUPLED RECEPTOR CHIMERAS

by

Arnold E. Ruoho
Michael K. Sievert
Mark P. Krebs
Andrew H. Geiser

The undersigned hereby certifies that this document is being deposited with the United States Postal Service today, September ____, 1999, by the "Express Mail" service, utilizing Express Mail label number EJ776142175US, addressed to: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Please indicate receipt of this application by returning the attached postcard with the official Patent and Trademark Office receipt and serial number stamped thereon.

Respectfully submitted,

A handwritten signature in cursive script that reads "Robin Miller".

BACTERIORHODOPSIN/G PROTEIN-COUPLED RECEPTOR CHIMERAS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial No. 60/098,950, filed September 3, 1998 which is incorporated by reference herein.

5

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was supported by United States
10 government funds in the form of grant NIH GM33138, awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

Signal transduction is an exquisite regulatory system
15 that affords rapid adjustment of intracellular functions and activities in response to changes in the extracellular environment. In multicellular eukaryotic organisms, signal transduction allows communication between various specialized cell types.

20 Guanine nucleotide-binding (G) protein-coupled receptors constitute a superfamily of receptors that are involved in regulating the function of virtually every cell in the human body. Currently, more than 250 types of G protein-coupled receptors have been identified,
25 including beta-adrenergic, serotonin, muscarinic acetylcholine, tachykinin, prostaglandin, and rhodopsin receptors. It is believed that this superfamily may contain thousands of members.

G protein-coupled receptors are heptahelical proteins
30 with seven transmembrane domains, three extracellular loops, and three intracellular loops, with the amino (N) terminus oriented extracellularly and the carboxy (C) terminus oriented intracellularly. The intracellular loop proximal to the C-terminus (loop 3) is highly variable in

length and is believed to confer specificity of interaction with G proteins.

When G protein-coupled receptors bind to specific ligands at the extracellular surface of the cell membrane, a conformational change in the G protein receptor is believed to occur. Ligand binding allows intracellular loop 3 to associate with an intracellular G protein. The interaction between the G protein-coupled receptor and the G protein triggers a cascade of intracellular events mediated by various downstream effectors, such as adenylate cyclase.

G protein-coupled receptors are critically important in regulating normal cellular functions and in maintaining health. These receptors also play an important role in the design and development of drug treatments for various diseases. Drugs that are either agonistic or antagonistic for ligands that bind to particular receptors have been developed to alter intracellular activities. For example, β blockers are a class of β adrenergic antagonists that are commonly used in the treatment of high blood pressure.

A number of diseases have been linked to mutations that interfere with the ability of a G protein-coupled receptor to bind to extracellular ligands. Diseases known to be caused by G-protein-coupled receptor mutations include, for example, retinitis pigmentosa, color blindness, nephrogenic diabetes insipidus, and hyperfunctioning thyroid adenomas. These diseases, which are either heritable or caused by somatic mutations, are refractory to treatment using agonists or antagonists. It is expected that the number of diseases attributable to mutations in G protein-coupled receptors will continue to grow as more is learned about this important family of receptors.

Several serious diseases are associated with the uncoupling of ligand binding to G protein-coupled receptors and activation of the signaling pathway. In other words, the signaling pathway is constitutively

activated in an agonist-independent manner. For example, Kaposi sarcoma-associated herpesvirus (KSHV/HHV8) encodes a G-protein coupled receptor that stimulates signaling pathways constitutively to induce transformation and
5 angiogenesis in KSHV-mediated oncogenesis. (Bais et al., Nature 391:86, 1998).

Agonists or antagonists of ligands that normally bind to G protein-coupled receptors are not effective in treating diseases caused by mutations that reduce or
10 prevent ligand binding to G protein-coupled receptors, or which result from the constitutive activation of signaling pathways in an agonist-independent manner. Treatment of these types of diseases depends upon the development of pharmaceuticals capable of altering the activation of
15 signaling pathways at a different level of control. We propose that this may be achieved by enhancing or reducing G protein interaction with a G protein-coupled receptor, thereby altering the activity of G proteins.

Progress in elucidating the mechanism by which G
20 proteins function in signal transduction has been impeded by difficulties associated with isolating G protein-coupled receptors in intact, functional form and in quantities sufficient to allow comprehensive studies to be conducted.

25 What is needed in the art is a method by which receptor proteins having a domain associated with G-protein activation can be isolated in quantities sufficient to allow screening of potential therapeutic agents.

30

BRIEF SUMMARY OF THE INVENTION

The development of efficacious drugs for treating diseases caused by errors in signaling pathways involving
35 G protein-coupled receptors would be facilitated by a method of obtaining sufficient amounts of protein comprising relevant G protein-coupled receptor sequences.

The ability to obtain a chimeric protein comprising at least a portion of intracellular loop 3 will facilitate screening of potential therapeutics for interaction with this critical domain.

5 The present invention includes a chimeric protein comprising a bacteriorhodopsin polypeptide sequence and a G protein-coupled receptor polypeptide sequence. Preferably, the protein comprises a bacteriorhodopsin scaffolding in which the amino acid sequence specifying
10 intracellular loop 3 of bacteriorhodopsin has been replaced by an amino acid sequence specifying at least a portion of intracellular loop 3 of a G protein-coupled receptor.

Another aspect of the present invention is a genetic
15 construct comprising a nucleotide sequence that encodes a bacteriorhodopsin/G-protein coupled receptor chimeric protein operably connected to a promoter.

Yet another aspect of the present invention is a method of preparing a bacteriorhodopsin/G protein-coupled
20 receptor chimeric protein comprising the steps of transforming an archaebacterium with a genetic construct comprising a nucleotide sequence that encodes a bacteriorhodopsin/G protein-coupled receptor chimeric protein, the nucleic acid sequence expressible in the
25 archaebacterium host, culturing the archaebacterium under suitable conditions of growth, and allowing expression of the chimeric protein.

It is an object of the present invention to provide a bacteriorhodopsin/G protein-coupled receptor protein
30 chimeric protein that may be used in further research into the role of G protein-coupled receptors in signal transduction and in drug development assays.

It is an advantage of the present invention that intact bacteriorhodopsin/G protein-coupled receptor
35 chimeric protein can be isolated in quantities sufficient to be used in high throughput assays of potential

therapeutics, as well as to obtain information about the structure of the replacement loop.

Other objects, features, and advantages of the present invention will be apparent upon review of the
5 specification and claims.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention makes it possible to obtain relatively large quantities of a chimeric,
10 membrane-associated fusion protein that primarily comprises largely bacteriorhodopsin amino acid sequence, in which an amino acid sequence that specifies a portion of the bacteriorhodopsin protein has been replaced by an amino acid sequence for the structurally analogous region
15 from a G-protein coupled receptor. The availability of chimeric bacteriorhodopsin/G-protein coupled receptor fusion proteins will facilitate studies designed to assess the role of various domains of G-protein coupled receptors in signal transduction, and facilitate identification of
20 potential therapeutic agents that are capable of interacting with the G protein-coupled receptor so as to alter signal transduction.

Bacteriorhodopsin is a membrane protein found in halophilic archaebacteria. Bacteriorhodopsin is
25 structurally very similar to the G protein-coupled receptors, in that it is a heptahelical protein with seven transmembrane domains, three extracellular loops, and three intracellular loops, with the amino (N) terminus oriented extracellularly and the carboxy (C) terminus
30 oriented intracellularly. However, bacteriorhodopsin is functionally distinct from the G protein-coupled receptors in that it functions as a proton "pump" in the transduction of light energy to chemical energy. Bacteriorhodopsin has been extremely well characterized,
35 in part because the protein forms a crystal structure in its native membrane environment, which facilitates purification and characterization.

U.S. Patent No. 5,641,650, incorporated by reference herein, discloses a method for producing a heterologous polypeptide by expressing a heterologous DNA sequence under the control of the transcriptional and translational regulatory sequences from a bacteriorhodopsin gene in halobacterium. Disclosed examples of heterologous proteins thus expressed include G protein-coupled receptors and G protein-coupled receptors lacking intracellular loop three.

Like bacteriorhodopsin, the chimeric fusion proteins produced by the method of the present invention are directed toward the membrane and are able to bind retinal, which suggests that these chimeras may have a crystal lattice structure comparable to that of bacteriorhodopsin. Preliminary comparative data obtained by ultraviolet and visible absorbance spectrophotometry (UV/VIS), circulator dichromism (CD), and sucrose density gradient isolation suggest that the chimeras may have a crystal structure similar to that of bacteriorhodopsin. Chimeric fusion proteins in which discrete portions of bacteriorhodopsin have been replaced with a specific domain from G protein-coupled receptor proteins will allow further investigation into the roles specific domains play in signal transduction.

Large quantities of G protein-coupled receptor intracellular loop 3 in a lattice structure were obtained in the form of chimeric fusion proteins in which the third loop of the bacteriorhodopsin protein was replaced with the third loop of any one of several G protein receptors.

A bacteriorhodopsin DNA fragment comprising the bacteriorhodopsin gene from *Halobacterium salinarium* was used to construct the chimeras as described below. The sequences of the DNA fragment and the putative amino acid sequence of bacteriorhodopsin are shown in SEQ ID NO: 1 and SEQ ID NO:2, respectively. The coding sequence for bacteriorhodopsin is found at base 394-1182 of sequence ID NO:1. Amino acid residues 1-13 of SEQ ID NO:2 constitute

a leader sequence that is cleaved to form the mature protein (amino acids 14-262 of SEQ ID NO:2). It is expected that any bacteriorhodopsin gene from any archaeobacterial species could be successfully employed in the practice of this invention. A bacteriorhodopsin gene of known sequence is preferable, because the sequence information facilitates selection of suitable restriction enzymes and design of oligonucleotide primers that can be used to construct a sequence encoding a chimeric protein.

10 *Halobacterium salinarium* strain MPK40, which was engineered to delete the bacteriorhodopsin gene (Krebs, et al. Proc. Natl. Acad. Sci. USA, 90:3986-3990, 1993; incorporated by reference herein) was used as a host strain for obtaining stable transformants comprising
15 chimeric protein coding sequences integrated into the archaeobacterial chromosome. In contrast to wild type *Halobacterium* strains, which produce purple colonies, MPK40 produce orange colonies. Transformants of MPK40 containing a chimeric bacteriorhodopsin/G protein-coupled
20 receptor gene were used to express the chimeric proteins.

It is expected that any other suitable archaeobacterial species or strain may serve as a host for expressing the chimeric proteins. A suitable archaeobacterial species or strain is one that is capable
25 of expressing a chimeric bacteriorhodopsin/G protein-coupled receptor gene to produce a chimeric bacteriorhodopsin/G protein-coupled receptor fusion protein. Preferably, the strain lacks a bacteriorhodopsin gene. It is well within the ability of one of skill in
30 the art to genetically engineer an archaeobacterial strain to delete a particular gene, such as the bacteriorhodopsin gene.

Plasmid pMPK85, which was used to develop the transformants, has an insertion sequence (ISH1) (SEQ ID
35 NO:45) that allows integration of the bacteriorhodopsin/G protein-coupled receptor gene into the archaeobacterial chromosome. It is envisioned that other IS elements may

be useful in obtaining stable integration of a bacteriorhodopsin/G protein-coupled receptor gene.

In designing the chimeric proteins of the present invention, *in vitro* mutagenesis was used to introduce a
5 unique *Bst*XI restriction site in transmembrane domain six. The native bacteriorhodopsin loop 3 coding region was removed by digestion with restriction endonucleases *Bst*XI and *Bsr*GI, which digests the bacteriorhodopsin (BOP) gene at a unique site in the coding region for transdomain 5.
10 One of skill in the art can appreciate that similar constructs could be prepared using engineered or native restriction sites other than *Bst*XI and *Bsr*GI. In addition to the transdomain 6 mutation, a *Bsu*36I site was created in transmembrane domain 7 using *in vitro* mutagenesis.
15 This restriction site may be used in constructing chimeras in which the carboxy terminus of bacteriorhodopsin is replaced with the carboxy terminus of a G protein-coupled receptor.

The mutations created in the bacteriorhodopsin gene
20 are silent, in that these mutations do not alter the amino acid sequence of the protein expression product. In designing the chimeric proteins of the present invention, one of skill in the art would recognize that an alteration in the primary sequence of a transmembrane domain could
25 result in perturbation of the higher order protein structure. It should be appreciated that it may be possible to make certain conservative substitutions in the amino acid sequence without affecting the secondary or tertiary structures.

30 In the examples below, chimeric proteins were constructed in which a portion of intracellular loop 3 of bacteriorhodopsin was replaced with the structurally analogous intracellular loop 3 region from bovine rhodopsin, adrenocorticotrophic hormone receptor,
35 adenosine A1 receptor, or human beta2 adrenergic receptor. It is reasonable to expect that the present invention

could be successfully practiced using a loop 3 region from any G protein-coupled receptor.

By "an intracellular loop 3 region" it is meant an amino acid sequence that includes at least a portion of an amino acid sequence corresponding to the amino acid sequence that forms intracellular loop three of a G protein-coupled receptor. Preferably, the intracellular loop three region is one that is able to alter the rate of GTP-GDP exchange from a G protein in an in vitro exchange assay.

The oligonucleotide sequences encoding G protein-coupled receptor sequences and used in the construction of the bacteriorhodopsin G protein-coupled receptor chimeras are preferably designed to optimize codon usage in *Halobacterium salinarium* so as to prevent pausing during translation and to maximize protein yields. However, it is expected that minor variations in sequence associated with nucleotide additions, deletions, and mutations, whether naturally occurring or introduced *in vitro* may not affect the expression of the chimeric protein. The scope of the present invention is intended to encompass minor variations in the chimeric protein sequence.

The chimeric proteins described below contain substantially all of the amino acid sequence of the wild type bacteriorhodopsin, with the exception for the deletion of intracellular loop 3 amino acid 171 to amino acid 179 of SEQ ID NO:2. The region corresponding to amino acids 171-179 of SEQ ID NO:2 was replaced with an intracellular loop 3 sequence from one of three G protein-coupled receptors. In each case, the G protein-coupled receptor loop 3 region was inserted between the threonine residue at position 170 of SEQ ID NO:2 (amino acid residue 157 of the mature bacteriorhodopsin) and the valine residue at position 180 of SEQ ID NO:2 (amino acid residue 167 of the mature bacteriorhodopsin).

It should be appreciated that it other portions of bacteriorhodopsin protein may be replaced with the

corresponding structurally analogous sequence from a G protein-coupled receptor. By a "structurally analogous sequence" it is meant a sequence that forms a region of the G protein-coupled receptor corresponding to the

5 bacteriorhodopsin region to be replaced (e.g., an intracellular or extracellular loop, the N-terminal or C-terminal region, or portions thereof). One wishing to substitute a portion of another region or regions of bacteriorhodopsin sequences with sequences from G protein-

10 coupled receptors could readily do so, using the teachings of the present invention and standard molecular biology techniques. By way of example, it may be advantageous to create a bacteriorhodopsin/G protein-coupled chimera comprising the intracellular loop 3 region and the C-

15 terminal region of a G protein-coupled receptor. This could be accomplished through the proper selection of restriction enzymes and primers, using the teachings and guidance provided herein.

The intracellular loop 3 of G protein-coupled

20 receptors is involved in promoting GTP-GDP exchange in G-proteins. The functionality of six different chimeric proteins in which the bacteriorhodopsin loop three region had been replaced with various regions of bovine rhodopsin intracellular loop three was evaluated by measuring the

25 rate of GTP-GDP exchange using transducin. Some, but not all, tested chimeric proteins were found to enhance GTP-GDP exchange relative to basal levels of exchange. This effect was inhibited by the presence of a high affinity analog of a peptide based on the C-terminus of the transducin alpha

30 subunit.

It is specifically envisioned that, in addition to G proteins, many other proteins will be able to interact with the loop 3 region of the chimeric fusion protein of the present invention. A brief list of proteins known to

35 interact with the loop 3 region of G protein-coupled receptor proteins includes protein 14-3-3 (J.Biol. Chem. 274(19):13462-9, 1999), which interacts with the alpha 2

adrenergic receptor; proteins Grb 2 and Nck (Biochemistry 37: 15726-36, 1998), which interact through SH3 interaction domains found on loop 3 of the dopamine D4 receptor; protein kinase A (Brain Research Bulletin 42:427-5 30, 1997), which phosphorylates cytoplasmic loop 3 of the alpha 1 adrenergic receptor; and rhodopsin kinase (J. Biol. Chem. 266: 12949-12955, 1991), which is activated by binding cytoplasmic loop 3 of rhodopsin).

It is reasonably expected that a bacteriorhodopsin/G
10 protein-coupled receptor protein in which the intracellular loop 3 region of bacteriorhodopsin is replaced by the structurally analogous loop 3 region from a G protein-coupled receptor may interact with a protein that interacts with the loop 3 region of the native G protein-coupled
15 receptor. One skilled in the art will appreciate that specific interaction between chimeric loop 3 polypeptides and proteins that interact with the loop 3 region of the native receptor can be evaluated by using specific assays. For example, the ability of protein kinase A to
20 phosphorylate a bacteriorhodopsin/G protein-coupled receptor protein comprising a portion of the loop three region of the alpha 1 adrenergic receptor could be determined by measuring phosphorylation of the chimeric protein by protein kinase A using radiolabeled ATP as a
25 substrate.

As demonstrated in the examples, chimeric bacteriorhodopsin/G protein-coupled receptor protein can be isolated intact in relatively large quantities. We expect that the availability of chimeric bacteriorhodopsin/G
30 protein-coupled receptor protein will facilitate evaluation of potential pharmaceutical agents that can enhance or reduce activation of G proteins. Additional advantages of these fusion proteins is enhanced protein stability of the G protein-coupled receptor and opportunities for obtaining
35 high resolution crystal structure information structure information, which may also facilitate drug development.

It is envisioned that the chimeric proteins of the present invention may be used in assays designed to evaluate interaction between potential therapeutic agents and the intracellular loop 3 of the G protein-coupled
5 receptor. Any suitable means for evaluating the interaction of a potential pharmaceutical molecule with the intracellular loop 3 could be employed. One could evaluate the ability of the potential pharmaceutical agent to
10 interact with intracellular loop 3 by incubating the agent with the protein under suitable conditions for a period of time sufficient to allow interaction to occur and evaluating the reaction mixture for the presence or absence of interaction. For example, interaction with loop three may alter the ability of the chimeric protein to promote
15 GTP-GDP exchange, which could be measured as described in the examples. The ability of an agent to alter the activity of an enzyme that is activated by interaction with loop 3 or which has loop 3 as a substrate could be evaluated by measuring the agent's effect on the enzymatic
20 reaction. Alternatively, the protein or test molecule could be detectably labeled to allow convenient detection of the interaction.

The following nonlimiting examples are intended to be purely illustrative.

25

EXAMPLES

Bacterial Strains and Culture Conditions

Halobacterium salinarium MPK40 was grown as previously described (Krebs, et al. *supra*). This strain lacks a bacteriorhodopsin gene, and therefore does not make purple membranes. Briefly, a single colony of *Halobacterium salinarium* MPK40 was used to inoculate 5 ml of culture medium (4.2 M NaCl, 0.08 M MgSO₄, 0.01M trisodium citrate, 0.026 M KCl, yeast extract (3 g/l), tryptone (5 g/l)) and cultured for 4 days at 37°C with 250 RPM shaking. For transformations, a 1-ml aliquot of the saturated starter culture was used to inoculate 50 ml of culture medium and grown for 20 hours at 37°C with shaking to an optical density of 0.5 at A₆₆₀. For protein purifications, a 5-ml saturated starter culture was used to inoculate a 1.8l volume of culture medium, which was cultured as described above.

Construction of Bacteriorhodopsin Chimeras

The plasmid pMPK85 (Krebs et al. *Biochemistry* 38:9023-9030, 1999), a derivative of MPK62 (Krebs, et al., *Proc. Natl. Acad. Sci. USA* 90:1987-1991, 1993), was used to construct bacteriorhodopsin-G protein receptor chimeric proteins. The pMPK85 plasmid contains a DNA fragment comprising a bacteriorhodopsin gene (BOP) inserted into the BamHI site of the multiple cloning region, as well as mevinolin and ampicillin resistance markers (Fig. 1). The aforementioned publications by Krebs et al. are incorporated by reference in their entirety.

To obtain large quantities of the BOP gene, the gene was subcloned into an *E. coli* vector using standard molecular biological techniques. Briefly, the BOP gene was excised from the parent vector by digesting pMPK85 with BamHI restriction endonuclease. A BamHI fragment containing the BOP gene was ligated to BamHI-linearized

pGEM 11ZF(+)vector (Promega Corp., Madison, WI), and the ligation mixture was used to transform Xl-1 Blue *E. coli* by electroporation. A transformant bearing a plasmid (designated pAHG) having the BOP insert was obtained. The
5 plasmid was purified using a Qiagen plasmid miniprep kit, followed by agarose gel purification.

Purified pAHG plasmid was mutated using the Gene Editor kit (Promega Corp.) to create silent mutations that resulted in novel restriction sites. A primer having the
10 sequence shown in SEQ ID NO:3 was used to create a *Bst*XI site on transmembrane domain 4. A primer having the sequence shown in SEQ ID NO:4 was used to create a *Bsu*36I site on transmembrane domain 7. A mutated plasmid (designated pAHGB) containing both restriction sites was
15 obtained.

To remove the region that encodes cytoplasmic loop 3 of BOP, pAHGB DNA was digested with *Bst*XI and *Bsr*GI. The BOP gene contains a native *Bsr*GI site on transmembrane domain 5. The doubly-digested pAHGB was then treated with
20 calf alkaline intestinal phosphatase and purified by agarose gel electrophoresis. Sequences encoding cytoplasmic loop 3 of bovine rhodopsin, adrenocorticotrophic hormone receptor, or adenosine A1 receptor were synthesized as described below (Generation of Loop 3
25 Inserts) and ligated to the large *Bst*XI-*Bsr*GI fragment from pAHGB to obtain pAHGBRI3,1; pAHGACTRI3,1; pAHGAAIAI3,1, respectively. Fragments containing sequences encoding the chimeric proteins comprising BOP and cytoplasmic loop 3 from a G protein receptor were ligated to *Bam*HI-digested
30 pMPK85 that was isolated from the BOP insert. The ligation mixture was introduced into *E. coli* by electroporation.

Transformants containing bacteriorhodopsin chimeric constructs having cytoplasmic loop 3 of bovine rhodopsin, adrenocorticotrophic hormone receptor, or adenosine A1
35 receptor were obtained and the plasmids were designated pMPKBRI3,1, pMPKACTRI3,1, and pMPKAAIAI3,1, respectively.

Because this plasmid has a low copy number, a Qiagen midi plasmid prep kit was used to purify plasmid DNA from transformants.

The BamHI inserts from these plasmids were ligated to
5 BamHI-linearized pAHG DNA, and the ligation mixture was
used to transform *H. salinarium* MPK40 strain. The plasmid
pAHG contains an insertion sequence ISH1 (SEQ ID NO:45)
that allows the chimeric gene to integrate into the
chromosome. Integration was confirmed by standard PCR
10 analysis. DNA sequences of the chimeric protein coding
regions were confirmed using the Big Dye sequencing system
at the University of Wisconsin Biotech Center.

Generation of Cytoplasmic Loop 3 Inserts

15 Synthetic DNA oligonucleotides were obtained from
Integrated DNA Technologies, Corp. The oligonucleotide
sequences were designed so as to obtain maximum codon usage
in *H. salinarium*, as discussed in Archea: A Laboratory
Manual, Rob, F.T., et al., Cold Spring Harbor, Appendix 4,
20 pp. 191-194, 1995).

The coding region for the bovine rhodospin cytoplasmic
loop 3 from V230 to M283 of bovine rhodopsin chimera was
obtained by combining SEQ ID NO: 3 and SEQ ID NO:4, which
have a 20 base pair region of complementarity, heating the
25 mixture to 98°C, and annealing by cooling the
oligonucleotides in a Perkin-Elmer 480 thermocycler at a
rate of 1.5°C/minute to 20 °C.

SEQ ID NO:5 Rhod I3,1 TopA:

30 5'ATCCTGTACGTGCTGTTCTTCGGGTTCAACGTCAAGGAGGCGGCGGCAGCAGCAG
GAGTCGGCGACGACGCAGAAGGCGGAGAAGGAGG 3'

and

SEQ ID NO:6 Rhod I3,1 BotA:

5'CGGGATACGCGGACCACAACACAACGGTAACGTTACGCAGTACTTTGAACGTGGATG
35 CGACCTCCATGCGCGTGACCTCCTTCTCCGCCTTCTGCG 3'

Sequences complementary to the *Bsr*GI restriction site in transmembrane domain 5 and to the *Bst*XI site in transmembrane domain 6 of the BOP gene were created by first treating the annealed oligonucleotide pairs with T4 DNA polymerase at 37°C for 10 minutes and then amplifying the sequences using two different sets of primers (SEQ ID NO:7, 8, 9, and 10) and *Pfu* polymerase in a PCR reaction.

Set 1:

10 SEQ ID NO:7 (NBBsrGITOPI): 5'-GTACATCCTGTACGTGCTGTTCTTCG-
3'
SEQ ID NO:8 (NBBstXIBOT2): 5'-ACGACGGGATACGCGGACC-3'

Set 2:

15 SEQ ID NO:9 (NBBsrGITOP3): 5'-ATCCTGTACGTGCTGTTCTTCG-3'
SEQ ID NO:10 (NBBstXIBOT4): 5'-CGGGATACGCGGACC-3'

PCR products were purified by agarose gel electrophoresis, combined into a single PCR tube, heated to 98°C, and annealed by cooling. The constructs thus generated have *Bst*XI and *Bsr*GI compatible ends that allow ligation to the bacteriorhodopsin gene in the proper orientation.

25 The coding region of cytoplasmic loop 3 (A200 to G217) of adrenocorticotrophic hormone receptor was prepared using synthetically prepared oligonucleotides SEQ ID NO:11 and SEQ ID NO:12, which have a 14-base pair region of complementarity.

30

SEQ ID NO:11 (ACTRTOPA):
5'ATCCTGTACGTGCTGTTCTTCGGGTTACCGCGCGCTCCCACACGCGCAAGATCTCC
ACGCTCCCGCGCGCAACATGAAGGG 3'
and

35

SEQ ID NO:12 (ACTRBOTA):
5'CGGGATACGCGGACCACAACACAACGGTAACGTTACGCAGTACTTTGAACGTGGATG
CGACGCCCTTCATGTTCG 3'

These oligonucleotides were treated in the same manner as those used in creating the bacteriorhodopsin-bovine rhodopsin chimera.

A coding sequence for the loop 3 region (E202 to S235) of the adenosine A1 receptor was obtained by combining SEQ ID NO: 13 and SEQ ID NO:14, which have a 27 base pair region of complementarity, heating the mixture to 98°C, and annealing the oligonucleotides in a Perkin-Elmer 480 thermocycler.

10 SEQ ID NO:13 (AAIATOPA):

5'GGGTTCACCGAGGTCTTCTACCTCATCCGCAAGCAGCTGGACAAGAAGGTCTCCGCG
TCCTCCGGCGACCCGCAAGTACTACGGCAAG 3'

and

SEQ ID NO:14 (AAIABOTA):

15 5'CACAACGGTAACGTTACGCAGTACTTTGAACGTGGATGCGACGGACTTCGCGATCTT
GAGCTCCTTGCCGTAGTACTTCTGCGGGTCGCC 3'

These oligos were treated in the same manner as those used in creating the bovine rhodopsin chimera.

An insert comprising a portion of the loop 3 region of
20 the human beta2 adrenergic receptor adenosine was obtained by PCR amplification as described above using primer pair B2BOT900 (SEQ ID NO:48) and B2TOP1 (SEQ ID NO:52).

The PCR product thus obtained was amplified by PCR using the primers B2TOPA1 (SEQ ID NO:50) and B2TOPB1 (SEQ
25 ID NO:53). This PCR product was then doubly digested with BSRG1 and BSTX1, and ligated to doubly digested pAHGB.

The coding sequence of the bacteriorhodopsin/beta 2 adrenergic receptor loop 3 is shown in SEQ ID NO:46 and SEQ ID NO:47. The beta2 adrenergic receptor portion of the
30 loop 3 coding sequence is found between amino acid residues 12-73 of SEQ ID NO:47.

Construction of additional loop 3 bovine rhodopsin chimeras

Nine additional bovine rhodopsin loop 3 inserts
35 (designated 3A-3I) were obtained using the indicated oligonucleotides as detailed below. The amino acid sequences of the inserts are described by the corresponding

sequences in native bovine rhodopsin. For example, insert 3A (InsQ225-M253) is 31 amino acid residues long and has the sequence corresponding to the amino acid residues 225 (glutamine)-253 (methionine) of bovine rhodopsin.

5 Inserts 3A-3D were constructed using SEQ ID NO: 15 and SEQ ID NO:16 in a first polymerase chain reaction to generate a single PCR product. The extension product was amplified by PCR using a second set of primers (shown below)to generate a final PCR product. These products were
10 digested with BstXI and BsrGI, and ligated to vector DNA linearized by treatment with BstXI and BsrGI.

The following primer sets were used:

15 **InsQ225-M253 (3A) :**

BOVI3_2top (SEQ ID NO:15)
BOVI3_2BOT (SEQ ID NO:16)
BOVI3_2-1TOBSRG1 (SEQ ID NO:17)
BOVI3_2+1TO_BSTX1 (SEQ ID NO:18)

20

InsY223-T251 (3B) :

BovI3_2top (SEQ ID NO:15)
BOVI3_2BOT (SEQ ID NO:16)
BOVI3_2+1TO_BSRG1 (SEQ ID NO:19)

25 BOVI3_2-1BSTX1 (SEQ ID NO:20)

InsY223-M253 (3C) :

BovI3_2top (15)
30 BOVI3_2BOT (SEQ ID NO:16)
BOVI3_2+1TO_BSTX1 (SEQ ID NO:18)
BOVI3_2+1TO_BSRG1 (SEQ ID NO:19)

InsQ225-T251 (3D) :

35 BovI3_2top (SEQ ID NO:15)
BOVI3_2BOT (SEQ ID NO:16)
BOVI3_2-1TOBSRG1 (SEQ ID NO:17)

BOVI3_2-1BSTX1 (SEQ ID NO:20)

InsG224-M253 (3E) :

BovI3_2top (SEQ ID NO:15)

5 BOVI3_2BOT (SEQ ID NO:16)

BOVBSRG1SITE (SEQ ID NO:21)

BOVBSRG1NOSITE (SEQ ID NO:22)

BOVI3_2+1TO_BSTX1 (SEQ ID NO:18)

10 InsG224-T251 (3F) :

BovI3_2top (SEQ ID NO:15)

BOVI3_2BOT (SEQ ID NO:16)

BOVBSRG1SITE (SEQ ID NO:21)

BOVBSRG1NOSITE (SEQ ID NO:22)

15 BOVI3_2-1BSTX1 (SEQ ID NO:20)

InsY223-R252 (3G) :

BovI3_2top (SEQ ID NO:15)

BOVI3_2BOT (SEQ ID NO:16)

20 BOVI3_2+1TO_BSRG1 (SEQ ID NO:19)

BOVBSTX1CSITE (SEQ ID NO:23)

BOVBSTX1NOC SITE (SEQ ID NO:24)

InsQ225-R252 (3H) :

25 BovI3_2top (SEQ ID NO:15)

BOVI3_2BOT (SEQ ID NO:16)

BOVI3_2-1TOBSRG1 (SEQ ID NO:17)

BOVBSTX1CSITE (SEQ ID NO:23)

BOVBSTX1NOC SITE (SEQ ID NO:24)

30

InsG224-R252 (3I) :

BovI3_2top (SEQ ID NO:15)

BOVI3_2BOT (SEQ ID NO:16)

BOVBSRG1SITE (SEQ ID NO:21)

35 BOVBSRG1NOSITE (SEQ ID NO:22)

BOVBSTX1CSITE (SEQ ID NO:23)

BOVBSTX1NOC SITE (SEQ ID NO:24)

For the construction of inserts 3E-3I, the following series of PCR reactions were performed. SEQ ID NO:15 and SEQ ID NO:16 were annealed and amplified by PCR to generate a single PCR product. This PCR product was PCR-amplified 5 two times using primers having either one site (BsrGI or BstXI) or both sides of the insert with both the ***SITE and ***NOSITE primers. Both of these second PCR products were digested with either BsrGI or BstXI, or neither. After digestion the PCR products were added together and 10 heated and reannealed to generate a site complementary to either the BsrGI or BstXI site. This reannealed insert was ligated into pAHD digested with BstXI and BsrGI.

By way of illustration, the 3H chimera was generated using the indicated primers as described below.

15

InsQ225-R252 (3H) :

BovI3_2top (SEQ ID NO:15)

BOVI3_2BOT (SEQ ID NO:16)

BOVI3_2-1TOBSRG1 (SEQ ID NO:17)

20 BOVBSTX1CSITE (SEQ ID NO:23)

BOVBSTX1NOC SITE (SEQ ID NO:24)

After generating the first PCR product for this insert using SEQ ID NO:15 and SEQ ID NO:16, the PCR product was 25 amplified in separate PCR reactions using the following sets of primers:

BOVI3_2-1TOBSRG1 (SEQ ID NO: 17)

BOVBSTX1CSITE (SEQ ID NO: 23)

30

and

BOVI3_2-1TOBSRG1 (SEQ ID NO:17)

BOVBSTX1NOC SITE (SEQ ID NO:24)

35

to obtain two second PCR products. The two second products were digested with BsrGI. The BsrGI-digested PCR products were combined, heated, and annealed, and then ligated directly to linearized pAHG vector DNA having 5 compatible ends. This approach was followed for inserts 3E-3I.

The coding sequence and amino acid sequence of each chimeric loop 3 region, including the N-terminal threonine residue and the C-terminal valine residue of bacteriorhodopsin, are provided. The coding sequences for inserts 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, and 3I correspond to SEQ ID NO: 25, 27, 29, 31, 33, 35, 37, 39, and 41, respectively; the corresponding amino acid sequences of the loop region inserts are presented in SEQ ID NO: 26, 28, 30, 32, 34, 36, 38, 40, and 42.

Transformation of *H. salinarium*

Transformation was carried out as previously described (Krebs, et al. *supra*). Briefly, a 10 ml aliquot of an MPK40 culture ($OD_{660} = 0.5$) was centrifuged at $3,000 \times g$ for 10 minutes. The cell pellet was resuspended in 1 ml of spheroplasting solution (2M NaCl, 27 mM KCl, 50 mM Tris-Cl, pH 8.5, 15% w/v sucrose). Aliquots (0.2 ml) of cells resuspended in spheroplasting solution were gently agitated with 10 uL of EDTA-spheroplasting solution (spheroplasting solution containing 0.5M EDTA). Plasmid DNA (1.5 ug) in a volume of approximately 3 uL was diluted with 13.5 uL of spheroplasting solution. The DNA sample was mixed with the 0.2 ml cells resuspended in spheroplasting solution and the mixture was incubated at room temperature for 5 minutes. A 225-uL aliquot of PEG-spheroplasting solution (60% (w/v) PEG 600 to spheroplasting solution) was added to each sample. After 20 minutes, 5 ml of CM sucrose (15% w/v of sucrose in culture medium) was added to the mixture, and the sample was centrifuged for 20 minutes at $5,000 \times g$. The pellet was resuspended in 10 ml of CM sucrose and incubated for 16 hours at $37^{\circ}C$ on a shaker at 250 RPM.

Aliquots of 100 uL of the sample were plated on Mevinolin-complemented agar culture medium plates. Transformants were transferred to 1 ml of culture media, diluted 100 fold, and 100 ul aliquots were plated on CM plates.

5 Stable transformants having the bacteriorhodopsin G-protein coupled protein gene integrated into the chromosome were evaluated for the production of a purple pigment by placing the CM plates under white lights for 48-72 hours, after which time purple colonies were observable. Purple
10 pigment production indicates that the bacteriorhodopsin is able to bind retinal. PCR analysis was used to confirm chimera gene insertion. Transformants comprising, bacteriorhodopsin/bovine rhodopsin bacteriorhodopsin/adrenocorticotropin releasing hormone receptor, or the
15 bacteriorhodopsin/human beta2 adrenergic receptor appeared purple, whereas transformants comprising the bacteriorhodopsin/A-1 adenosine chimera appeared orange. These results indicate that the stable transformants comprising the bacteriorhodopsin/bovine rhodopsin chimera
20 or the bacteriorhodopsin/adrenocortico-tropin releasing hormone receptor chimera form purple membranes having a chromophore similar to that of bacteriorhodopsin.

Purification of bacteriorhodopsin/G-protein-coupled
25 **receptor chimeric protein**

Chimeric bacteriorhodopsin was purified from a transformant comprising the bacteriorhodopsin/bovine rhodopsin chimera in essentially the same way that native bacteriorhodopsin is purified from archaebacterial species
30 (4). Briefly, a single purple colony was used to inoculate 5 ml of culture media and cultured for 4 days at 37°C with shaking at 250 RPM. This culture was used to inoculate 1.8 L of culture medium, and the culture was grown for 5 days in an illuminated shaker (wavelength 570 nm) at 40°C
35 and 250 RPM. Cells were pelleted by centrifugation at 7,000 x g for 30 minutes and resuspended in 18 ml of water, and treated with 500 units of DNaseI. This

preparation was dialyzed against water for 10 hours with constant stirring. The dialysate was then centrifuged at 23,000 x g for 40 minutes. The pellets were resuspended in 2 ml of 5 mM NaCl, and placed over continuous sucrose 5 gradients (30-58%). The gradients were centrifuged in a swinging bucket rotor at 27,000 x g for 17 hours at 15°C. A purple band that migrated near the bottom of the centrifuge tube was collected. Typically, this purification procedure yields between 5 and 10 milligrams 10 of pure protein.

Characterization of Bacteriorhodopsin-Rhodopsin Chimera

The protein was characterized by ultraviolet and visible absorbance spectrophotometry (UV/VIS), circulator 15 dichromism (CD), and SDS-PAGE. The results of SDS-PAGE size estimates were consistent with the predicted molecular weight based on the deduced amino acid sequence.

A comparison of the UV/vis spectra from the bacterio- rhodopsin/bovine rhodopsin chimeric protein spectra reveals 20 very similar spectra, suggesting that the chimeric protein has the same chromophore (retinal) as the native bacteriorhodopsin that the chimeras may have the proper lattice formation.

The ability of the chimeric protein to promote GTP-GDP 25 exchange was evaluated for each of three BR-rhodopsin loop 3 chimeras by evaluating the effect of the chimera on the rate of GDP-GTP exchange on transducin using a GTPγS uptake assay, as described below.

Chimeric or bacteriorhodopsin protein (1.7 uM) was 30 incubated with 0.8 uM holotransducin with 10 nM ³⁵S radiolabeled GTPγS in buffer (20 mM Hepes (pH 7.5), 5 mM MgCl₂, 1mM DTT, .1 mM EDTA, and 20 mM NaCl). The reaction was incubated at 30°C for 5, 10, 15 or 30 minutes. The reaction was terminated by transferring aliquots of the 35 reaction mixture to nitrocellulose filters (0.45 um) prewetted with wash buffer (10 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA) and placed on a vacuum

manifold. The filters were then washed with 8 ml of wash buffer. The filters were then placed overnight in scintillation vials filled with 20 ml of scintillation cocktail. The activity of the vials was counted by a
 5 Liquid Scintillation Counter. For the BR-Rhodopsin loop 3 chimeras, in which the rhodopsin intracellular loop 3 replaced the native BR intracellular loop 3 and was fused from T157 on BR TM 5 to V167 on BR TM 6, activity was determined by an increased uptake of GTPS over basal
 10 exchange by transducin alone.

<u>Specific counts (Lattice Form) (at 30 min)</u>														
15	Rhodopsin Insert Y223 to M253 (3C)	262331												
	Rhodopsin Insert Y223 to R252 (3G)	182530												
	Rhodopsin Insert Q225 to R252 (3H)	193374												
	BR	117859												
	Basal Exchange of Transducin	164750												
20	<table> <tr> <th><u>Activation (Fold increase)</u></th><th><u>over BR</u></th><th><u>over Basal</u></th></tr> <tr> <td>Rhodopsin Insert Y223 to M253 (3C)</td><td>2.22</td><td>1.6</td></tr> <tr> <td>Rhodopsin Insert Y223 to R252 (3G)</td><td>1.55</td><td>1.1</td></tr> <tr> <td>Rhodopsin Insert Q225 to R252 (3H)</td><td>1.6</td><td>1.17</td></tr> </table>		<u>Activation (Fold increase)</u>	<u>over BR</u>	<u>over Basal</u>	Rhodopsin Insert Y223 to M253 (3C)	2.22	1.6	Rhodopsin Insert Y223 to R252 (3G)	1.55	1.1	Rhodopsin Insert Q225 to R252 (3H)	1.6	1.17
<u>Activation (Fold increase)</u>	<u>over BR</u>	<u>over Basal</u>												
Rhodopsin Insert Y223 to M253 (3C)	2.22	1.6												
Rhodopsin Insert Y223 to R252 (3G)	1.55	1.1												
Rhodopsin Insert Q225 to R252 (3H)	1.6	1.17												

25 A time course study was next performed for Rhodopsin Insert Y223 to M253 (3C)

<u>Specific counts (Lattice Form)</u>		<u>5</u>	<u>10</u>	<u>15 min</u>
30				
	Rhodopsin Insert	26273	51423	66566
	(Y223 to M253(3C))			
	Basal Exchange	16825	26325	33653

35		
	<u>Activation by 3C (x 100%)</u>	<u>Over Basal Exchange</u>
	5 Minutes	1.56

10 Minutes	1.95
15 Minutes	1.97

Additionally, the GTPyS exchange assay was repeated in the presence of varying concentrations of either a peptide that has been shown to inhibit transducin activation by rhodopsin ("high affinity analog"), or a random peptide.

The "high affinity analog" is the C-terminal portion of the transducin alpha subunit that competitively inhibits binding of transducin to G protein-coupled receptor protein. The high affinity analog has the sequence V-L-E-D-L-K-S-C-G-L-F-G (SEQ ID NO:43) (J.Biol.Chem. 271 pp. 361-366, 1996).

The random peptide consists of the sequence S-S-V-F-L-V-V-D-R-S-R (SEQ ID NO:44).

The addition of the high affinity analog at a final concentration of 8 uM gave the following results.

<u>Specific counts (Lattice Form)</u>	<u>10 Minutes</u>
Rhodopsin Insert Y223 to M253 (3C)	51423
Basal Exchange	26325
3C + 8 uM HIGH AFFINITY ANALOG	42455
Basal Exchange + 8 uM H.A.A.	23154
3C + 8 uM Random Peptide	47516
Basal Exchange + 8 uM Random Peptide	23868

% Inhibition of GTPyS Uptake (Controlled for Peptide inhibition of Basal Exchange)

8 uM High affinity analog	23%
8 uM Random peptide	5.8%

The second inhibition experiment used 50 uM of the high affinity analog.

<u>SPECIFIC COUNTS: (Lattice Form)</u>	<u>10 Minutes</u>
Rhodopsin Insert Y223 to M253 (3C)	93448
Basal Exchange	49988
3C + 50 uM H.A.A.	46892
5 Basal Exchange + 50 uM H.A.A.	33888
3C + 50 uM Random Peptide	35568

% Inhibition of GTPyS Uptake (Controlled for Peptide inhibition of Basal Exchange)

10

50 uM HIGH AFFINITY ANALOG	70%
50 uM Random peptide	30%

The next set of experiments was designed to measure the effects of GDP concentration on the basal exchange of GTPyS by holotransducin.

<u>Specific counts (Lattice Form)</u>	<u>10 Minutes</u>
20 Rhodopsin Insert Y223 to M253 100uM GDP	12577
Basal Exchange + 100 uM GDP	7502
Basal Exchange + 50 uM GDP	11061
Basal Exchange + 10 uM GDP	22885
Basal Exchange + 0 uM GDP	51307

25

Activation (3C) ($\times 10^0$) over Basal Exchange

Rhodopsin Insert Y223 to M253 100 μ M GDP	1.67
---	------

30 The following experiments repeated the study of GTPyS exchange with a new culture of the BR-Rhodopsin chimera for Rhodopsin Insert Y223 (3C) as well as three new chimeras.

35 <u>Specific counts (Lattice Form)</u>	<u>10 Minutes</u>
Rhodopsin Insert Y223 to T251 (3B)	62759

Rhodopsin Insert Y223 to M253 (3C)	77125
Rhodopsin Insert Q225 to T251 (3D)	53448
Rhodopsin Insert G224 to M253 (3E)	78433
Basal Exchange	53565

5

<u>Activation (X 100%)</u>	<u>Over Basal</u>
<u>Exchange</u>	

Rhodopsin Insert Y223 to T251 (3B)	1.17
10 Rhodopsin Insert Y223 to M253 (3C)	1.44
Rhodopsin Insert Q225 to T251 (3D)	1.0
Rhodopsin Insert G224 to M253 (3E)	1.46

15 The present invention is not limited to the exemplified embodiments, but is intended to encompass all such modifications and variations as come within the scope of the following claims.

20

25

CLAIMS

We claim:

5

1. A chimeric fusion protein comprising a bacteriorhodopsin protein amino acid sequence in which at least a portion of the protein is replaced with the structurally analogous region of a G protein-coupled
10 receptor protein.

2. The protein of claim 1, wherein the protein comprises substantially all of the amino acid sequence of bacteriorhodopsin except the intracellular loop 3 domain,
15 wherein the intracellular loop 3 domain of bacteriorhodopsin is replaced by at least a portion of the intracellular loop 3 domain of a G protein-coupled receptor protein.

3. The chimeric protein of claim 2, wherein the
20 intracellular loop 3 domain region corresponding to amino acid residues 171-179 of SEQ ID NO:2 is replaced with at least a portion of the intracellular loop 3 domain of a G protein-coupled receptor protein.

25 4. The chimeric protein of claim 2, wherein the protein is able to alter the rate of GTP-GDP exchange on a G protein *in vitro*.

5. The chimeric protein of claim 4, wherein the rate
30 of GTP-GDP exchange is increased.

6. A polynucleotide sequence encoding the chimeric fusion protein of claim 1.

35 7. A genetic construct comprising the polynucleotide sequence of claim 6, the polynucleotide sequence operably connected to a promoter sequence.

8. An archaebacterium comprising the genetic construct of claim 7, wherein the polynucleotide sequence of the construct is expressible in the archaebacterium.

5 9. The archaebacterium of claim 8, wherein the archaebacterium is characterized by reduced expression of wild type bacteriorhodopsin.

10 10. The archaebacterium of claim 8, wherein the genetic construct is integrated into the archaebacterium chromosome.

11. A method of producing a bacteriorhodopsin/G protein-coupled receptor chimeric fusion protein comprising
15 the step culturing an archaebacterium comprising a genetic construct having a polynucleotide sequence that encodes a chimeric fusion protein having bacteriorhodopsin protein amino acid sequence in which at least a portion of the protein is replaced with the structurally analogous region
20 of a G protein-coupled receptor protein, the polynucleotide sequence operably connected to a promoter sequence functional in the archaebacterium, wherein the polynucleotide sequence of the construct is expressible in the archaebacterium, under suitable conditions and for a
25 period of time sufficient to allow expression of the chimeric fusion protein.

12. The method of claim 11, further comprising the step of partially purifying the chimeric fusion protein.
30

35

13. A method of testing a molecule for its ability to interact with the intracellular loop 3 of a G protein-coupled receptor comprising the steps of:

- (a) reacting a chimeric fusion protein of comprising a
5 substantially all of the bacteriorhodopsin protein amino acid sequence amino acid sequence except the intracellular loop 3 domain, wherein the intracellular loop 3 domain of bacteriorhodopsin is replaced by at least a portion of the intracellular loop 3 domain of a G protein-coupled receptor
10 protein with a test molecule under suitable reaction conditions for a period of time sufficient to allow interaction between the molecule and the protein; and
- (b) detecting presence or absence of interaction between the protein and the test molecule in the reaction
15 mixture.

14. The method of claim 13, wherein the chimeric fusion protein of step (a) is able to promote GTP-GDP exchange on a G protein in vitro, and wherein the detecting
20 step (b) includes an *in vitro* GTP-GDP exchange assay.

ABSTRACT

Disclosed is a chimeric protein comprising a bacteriorhodopsin polypeptide sequence and a G protein-coupled receptor sequence. Also disclosed is a genetic construct comprising encoding a chimeric bacteriorhodopsin polypeptide sequence and a G protein-coupled receptor sequence. The chimeric protein of the present invention can be used to evaluate various agents for the ability to interact with loop 3 of a G protein-coupled receptor.

15

20

25

30

35

Please type a plus sign (+) inside this box ☐

Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

0010/PTO Rev. 6/95 U.S. Department of Commerce Patent and Trademark Office DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION Declaration OR Declaration <input checked="" type="checkbox"/> Submitted <input type="checkbox"/> Submitted after with Initial Filing Initial Filing	Attorney Docket Number	960296.95581
	First Named Inventor	Arnold E. Ruoho
	COMPLETE IF KNOWN	
	Application Number	
	Filing Date	
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BACTERIORHODOPSIN/ G PROTEIN-COUPLED RECEPTOR CHIMERAS

(Title of the Invention)

the specification of which

☒ is attached hereto

OR

☐ was filed on (MM/DD/YY)

as United States Application Number or PCT International

Application Number

and was amended on (MM/DD/YY)

(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YY)	Priority Not Claimed	Certified YES	Copy Attached? NO
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.
60/09850	09/03/98	

Please type a plus sign (+) inside this box ☐

DECLARATION

Page 2

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith:

<input type="checkbox"/> Firm Name		Customer Number or label	
OR			
<input checked="" type="checkbox"/> List attorney(s) and/or agent(s) name and registration number below			

Name	Registration Number	Name	Registration Number
Thad F. Kryshak	19,428	Gregory A. Nelson	30,577
Neil Hamilton	19,869	Keith M. Baxter	31,233
Thomas W. Ehrmann	20,374	John D. Franzini	31,356
Barry E. Sammons	25,608	Joseph W. Bain	34,290
J. Rodman Steele	25,931	Robert J. Sacco	35,667
Nicholas J. Seay	27,386	Jean C. Baker	35,433
George E. Haas	27,642	David G. Ryser	35,407
Michael J. McGovern	28,326	Bennett J. Berson	37,094
Carl R. Schwartz	29,437	Michael A. Jaskolski	37,551
		Jill A. Fahrlander	42,518

☐ Additional attorney(s) and/or agents named on a supplemental priority sheet attached hereto

Please direct all correspondence to ☐ Customer Number or label ☐ OR ☒ Fill in correspondence address below

Name	Fahrlander, Jill A.		
Address	Quarles & Brady LLP		
Address	P O Box 2113		
City	Madison	State	WI
Zip	53701-2113		
Country	US	Telephone	608/251-5000
Fax	608/251-9166		

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of Sole or First Inventor:				A petition has been filed for this unsigned inventor			
Given Name	Arnold	Middle Initial	E.	Family Name	Ruoho		
Suffix, e.g. Jr.							
Inventor's Signature					Date		
Residence: City	Madison	State	WI	Country	US	Citizenship	US
Post Office Address	1209 Brookwood Road						
Post Office Address							
City	Madison	State	WI	Zip	53711	Country	US
Applicant Authority							
<input type="checkbox"/> Additional inventors are being named on supplemental sheet(s) attached hereto							

Please type a plus sign (+) inside this box ☐

DECLARATION					ADDITIONAL INVENTOR(S) Supplemental Sheet				
Name of Additional Joint Inventor, if any:					A petition has been filed for this unsigned inventor				
Given Name	Michael	Middle Initial	K.	Family Name	Sievert	Suffix e.g. Jr.			
Inventor's Signature						Date			
Residence: City		Madison		State	WI	Country		US	
Post Office Address		5319 Brody Drive #104							
Post Office Address									
City	Madison	State	WI	Zip	53705	Country		US	
					Applicant Authority				
Name of Additional Joint Inventor, if any:					A petition has been filed for this unsigned inventor				
Given Name	Mark	Middle Initial	P.	Family Name	Krebs	Suffix e.g. Jr.			
Inventor's Signature						Date			
Residence: City		Madison		State	WI	Country		US	
Post Office Address		10-B University Houses							
Post Office Address									
City	Madison	State	WI	Zip	53705	Country		US	
					Applicant Authority				
Name of Additional Joint Inventor, if any:					A petition has been filed for this unsigned inventor				
Given Name	Andrew	Middle Initial	H.	Family Name	Geiser	Suffix e.g. Jr.			
Inventor's Signature						Date			
Residence: City		Madison		State	WI	Country		US	
Post Office Address		626 Langdon Street #904							
Post Office Address									
City	Madison	State	WI	Zip	53703	Country			
					Applicant Authority				
Name of Additional Joint Inventor, if any:					A petition has been filed for this unsigned inventor				
Given Name			Middle Initial			Family Name			Suffix e.g. Jr.
Inventor's Signature						Date			
Residence: City				State		Country			
Post Office Address									
Post Office Address									
City			State		Zip			Country	
					Applicant Authority				

Additional inventors are being named on supplemental sheet(s) attached hereto

SEQUENCE LISTING

<110> Ruoho, Arnold E.
Geiser, Andrew H.
Krebs, Mark
Sievert, Mike

<120> BACTERIORHODOPSIN/G PROTEIN-COUPLED RECEPTOR CHIMERAS

<130> 960296.95581

<140>

<141>

<160> 12

<170> PatentIn Ver. 2.0

<210> 1

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide

<400> 1

cgcgatatcca gtcgtgtggc

20

<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide

<400> 2

cctcctgagg agtcgtgcga

20

<210> 3

<211> 91

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide

<400> 3

atcctgtacg tgctgttctt cgggttcacc gtcaaggagg cggcggcgca gcagcaggag 60
tcggcgacga cgcagaaggc ggagaaggag g 91

SEQUENCE LISTING

<110> Ruoho, Arnold E.
Geiser, Andrew H.
Krebs, Mark
Sievert, Mike

<120> BACTERIORHODOPSIN/G PROTEIN-COUPLED RECEPTOR CHIMERAS

<130> 960296.95581

<140>

<141>

<150> 60/098950

<151> 1998-09-03

<160> 53

<170> PatentIn Ver. 2.0

<210> 1

<211> 1626

<212> DNA

<213> Halobacterium salinarium

<220>

<221> CDS

<222> (394) .. (1182)

<400> 1

```

ggatccgacg tgaagatggg gctcccgatg ggtgcaaccg tgaagtcctg cacggctgcg 60
tcacgacagg agccgaccag cgacaccag aaggtgcaaa cgggtgagtg ccgcaacgat 120
cacgagtttt tcgtgcgctt cgagtggtaa cacgcgtgca cgcacgact tcaccgcggg 180
tgtttcgacg ccagccggcc gttgaaccag caggcagcgg gcatttcaca gccgctgtgg 240
cccacacact cggtgggggtg cgctatcttg gtatgggttg gaatccgcgt gtcgggtccg 300
tgtctgacgg ttcacggtc taaattccgt caccagcgta ccatactgat tgggtcgtag 360
agttacacac atatcctcgt taggtactgt tgc atg ttg gag tta ttg cca aca 414
                               Met Leu Glu Leu Leu Pro Thr
                               1               5

gca gtg gag ggg gta tcg cag gcc cag atc acc gga cgt ccg gag tgg 462
Ala Val Glu Gly Val Ser Gln Ala Gln Ile Thr Gly Arg Pro Glu Trp
      10               15               20

```

atc tgg cta gcg ctc ggt acg gcg cta atg gga ctc ggg acg ctc tat	510
Ile Trp Leu Ala Leu Gly Thr Ala Leu Met Gly Leu Gly Thr Leu Tyr	
25 30 35	
ttc ctc gtg aaa ggg atg ggc gtc tcg gac cca gat gca aag aaa ttc	558
Phe Leu Val Lys Gly Met Gly Val Ser Asp Pro Asp Ala Lys Lys Phe	
40 45 50 55	
tac gcc atc acg acg ctc gtc cca gcc atc gcg ttc acg atg tac ctc	606
Tyr Ala Ile Thr Thr Leu Val Pro Ala Ile Ala Phe Thr Met Tyr Leu	
60 65 70	
tcg atg ctg ctg ggg tat ggc ctc aca atg gta ccg ttc ggt ggg gag	654
Ser Met Leu Leu Gly Tyr Gly Leu Thr Met Val Pro Phe Gly Gly Glu	
75 80 85	
cag aac ccc atc tac tgg gcg cgg tac gct gac tgg ctg ttc acc acg	702
Gln Asn Pro Ile Tyr Trp Ala Arg Tyr Ala Asp Trp Leu Phe Thr Thr	
90 95 100	
ccg ctg ttg ttg tta gac ctc gcg ttg ctc gtt gac gcg gat cag gga	750
Pro Leu Leu Leu Leu Asp Leu Ala Leu Leu Val Asp Ala Asp Gln Gly	
105 110 115	
acg atc ctt gcg ctc gtc ggt gcc gac ggc atc atg atc ggg acc ggc	798
Thr Ile Leu Ala Leu Val Gly Ala Asp Gly Ile Met Ile Gly Thr Gly	
120 125 130 135	
ctg gtc ggc gca ctg acg aag gtc tac tcg tac cgc ttc gtg tgg tgg	846
Leu Val Gly Ala Leu Thr Lys Val Tyr Ser Tyr Arg Phe Val Trp Trp	
140 145 150	
gcg atc agc acc gca gcg atg ctg tac atc ctg tac gtg ctg ttc ttc	894
Ala Ile Ser Thr Ala Ala Met Leu Tyr Ile Leu Tyr Val Leu Phe Phe	
155 160 165	
ggg ttc acc tcg aag gcc gaa agc atg cgc ccc gag gtc gca tcc acg	942
Gly Phe Thr Ser Lys Ala Glu Ser Met Arg Pro Glu Val Ala Ser Thr	
170 175 180	
ttc aaa gta ctg cgt aac gtt acc gtt gtg ttg tgg tcc gcg tat ccc	990
Phe Lys Val Leu Arg Asn Val Thr Val Val Leu Trp Ser Ala Tyr Pro	
185 190 195	
gtc gtg tgg ctg atc ggc agc gaa ggt gcg gga atc gtg ccg ctg aac	1038
Val Val Trp Leu Ile Gly Ser Glu Gly Ala Gly Ile Val Pro Leu Asn	
200 205 210 215	
atc gag acg ctg ctg ttc atg gtg ctt gac gtg agc gcg aag gtc ggc	1086
Ile Glu Thr Leu Leu Phe Met Val Leu Asp Val Ser Ala Lys Val Gly	
220 225 230	
ttc ggg ctc atc ctc ctg cgc agt cgt gcg atc ttc ggc gaa gcc gaa	1134
Phe Gly Leu Ile Leu Leu Arg Ser Arg Ala Ile Phe Gly Glu Ala Glu	
235 240 245	

Arg Pro Glu Val Ala Ser Thr Phe Lys Val Leu Arg Asn Val Thr Val
180 185 190

Val Leu Trp Ser Ala Tyr Pro Val Val Trp Leu Ile Gly Ser Glu Gly
195 200 205

Ala Gly Ile Val Pro Leu Asn Ile Glu Thr Leu Leu Phe Met Val Leu
210 215 220

Asp Val Ser Ala Lys Val Gly Phe Gly Leu Ile Leu Leu Arg Ser Arg
225 230 235 240

Ala Ile Phe Gly Glu Ala Glu Ala Pro Glu Pro Ser Ala Gly Asp Gly
245 250 255

Ala Ala Ala Thr Ser Asp
260

<210> 3
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 3
cgcgatatcca gtcgtgtggc 20

<210> 4
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 4
cctcctgagg agtcgtgcga 20

<210> 5
<211> 91
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 5
atcctgtacg tgctgttctt cgggttcacc gtcaaggagg cggcggcgca gcagcaggag 60
tcggcgacga cgcagaaggc ggagaaggag g 91

<210> 6
<211> 96
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 6
cgggatacgc ggaccacaac acaacggtaa cgttacgcag tactttgaac gtggatgcga 60
cctccatgcg cgtgacctcc ttctccgcct tctgcg 96

<210> 7
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 7
gtacatcctg tacgtgctgt tcttcg 26

<210> 8
<211> 19
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 8
acgacgggat acgcgacc 19

<210> 9
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 9
atcctgtacg tgctgttctt cg 22

<210> 10
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:oligonucleotide
 primer

<400> 10
 cgggatacgc ggacc 15

<210> 11
 <211> 83
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:oligonucleotide
 primer

<400> 11
 atctgtacg tgctgttctt cggttcacc gcgcgtccc acacgcgcaa gatctccacg 60
 ctcccgcgcg cgaacatgaa ggg 83

<210> 12
 <211> 75
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:oligonucleotide
 primer

<400> 12
 cgggatacgc ggaccacaac acaacggtaa cgttacgcag tactttgaac gtggatgcga 60
 cgcccttcac gttcg 75

<210> 13
 <211> 89
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:oligonucleotide
 primer

<400> 13
 gggttcaccg aggtcttcta ctcacccgc aagcagctga caagaaggtc tccgcgtcct 60
 ccggcgaccc gcagaagtac tacggcaag 89

<210> 14
 <211> 90
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:oligonucleotide
 primer

<400> 14

cacaacggta acgttacgca gtactttgaa cgtggatgcg acggacttcg cgatcttgag 60
ctccttgccg tagtacttct gcgggtcgcc 90

<210> 15

<211> 84

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 15

gggttcaccg gccagctcgt cttcacggtc aaggaggcgg cggcgagca gcaggagtcg 60
gcgacgacgc agaaggcgga gaag 84

<210> 16

<211> 90

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 16

ggaccacaac acaacggtaa cgttacgcag tactttgaac gtggatgcga cgcggtgac 60
ctccttctcc gccttctgcg tcgtcgccga 90

<210> 17

<211> 68

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 17

tttgatcatg tacatcctgt acgtgctgtt cttcgggttc accagctcg tcttcacggt 60
caaggagg 68

<210> 18

<211> 100

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 18
gctgccgatac agccacacga ctggatacgc ggaccacaac acaacggtaa cgttacgcag 60
tactttgaac gtggatgcga ccatgcgcgt gacctccttc 100

<210> 19
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 19
tttgtacatg tacatcctgt acgtgctgtt cttcgggttc acctacggcc agctcgtctt 60
cacggccaag gagg 74

<210> 20
<211> 100
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 20
gctgccgatac agccacacga ctggatacgc ggaccacaac acaacggtaa cgttacgcag 60
tactttgaac gtggatgcga ccgtgacctc cttctccgcc 100

<210> 21
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 21
gtacatcctg tacgtgctgt tcttcgggtt caccggc 37

<210> 22
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 22
atcctgtacg tgctgttctt cgggttcacc ggc 33

<210> 23
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 23
acgacgggat acgcggacca caacacaacg g 31

<210> 24
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 24
cgggatacgc ggaccacaac acaacgg 27

<210> 25
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:chimeric loop 3
sequence

<220>
<221> CDS
<222> (1)..(93)

<400> 25
acc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag cag gag 48
Thr Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln Glu
1 5 10 15

tcg gcg acg acg cag aag gcg gag aag gag gtc acg cgc atg gtc 93
Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Met Val
20 25 30

<210> 26
<211> 31
<212> PRT
<213> Artificial Sequence

<400> 26
Thr Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln Glu
1 5 10 15

Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Met Val
20 25 30

<210> 27
 <211> 93
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(93)

<220>
 <223> Description of Artificial Sequence:chimeric loop 3
 sequence

<400> 27
 acc tac ggc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag 48
 Thr Tyr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln
 1 5 10 15
 cag gag tcg gcg acg acg cag aag gcg gag aag gag gtc acg gtc 93
 Gln Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Val
 20 25 30

<210> 28
 <211> 31
 <212> PRT
 <213> Artificial Sequence

<400> 28
 Thr Tyr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln
 1 5 10 15
 Gln Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Val
 20 25 30

<210> 29
 <211> 99
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:chimeric loop 3
 sequence

<220>
 <221> CDS
 <222> (1)..(99)

<400> 29
 acc tac ggc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag 48
 Thr Tyr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln
 1 5 10 15
 cag gag tcg gcg acg acg cag aag gcg gag aag gag gtc acg cgc atg 96
 Gln Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Met
 20 25 30

gtc 99
 Val

<210> 30
<211> 33
<212> PRT
<213> Artificial Sequence

<400> 30
Thr Tyr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln
1 5 10 15
Gln Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Met
20 25 30

Val

<210> 31
<211> 87
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)..(90)

<220>
<223> Description of Artificial Sequence:chimeric loop 3
sequence

<400> 31
acc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag cag gag 48
Thr Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln Glu
1 5 10 15
tcg gcg acg acg cag aag gcg gag aag gag gtc acg gtc 87
Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Val
20 25

<210> 32
<211> 29
<212> PRT
<213> Artificial Sequence

<400> 32
Thr Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln Glu
1 5 10 15
Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Val
20 25

<210> 33
<211> 96
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)..(96)

<220>

<223> Description of Artificial Sequence:chimeric loop 3
sequence

<400> 33

acc ggc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag cag 48
Thr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln
1 5 10 15

gag tcg gcg acg acg cag aag gcg gag aag gag gtc acg cgc atg gtc 96
Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Met Val
20 25 30

<210> 34

<211> 32

<212> PRT

<213> Artificial Sequence

<400> 34

Thr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln
1 5 10 15

Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Met Val
20 25 30

<210> 35

<211> 90

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(90)

<220>

<223> Description of Artificial Sequence:chimeric loop 3
sequence

<400> 35

acc ggc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag cag 48
Thr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln
1 5 10 15

gag tcg gcg acg acg cag aag gcg gag aag gag gtc acg gtc 90
Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Val
20 25 30

<210> 36

<211> 30

<212> PRT

<213> Artificial Sequence

<400> 36

Thr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln
1 5 10 15

Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Val
 20 25 30

<210> 37

<211> 96

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(96)

<220>

<223> Description of Artificial Sequence:chimeric loop 3
 sequence

<400> 37

acc tac ggc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag 48
 Thr Tyr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln
 1 5 10 15

cag gag tcg gcg acg acg cag aag gcg gag aag gag gtc acg cgc gtc 96
 Gln Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Val
 20 25 30

<210> 38

<211> 32

<212> PRT

<213> Artificial Sequence

<400> 38

Thr Tyr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln
 1 5 10 15

Gln Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Val
 20 25 30

<210> 39

<211> 90

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(90)

<220>

<223> Description of Artificial Sequence:chimeric loop 3
 sequence

<400> 39

acc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag cag gag 48
 Thr Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Glu
 1 5 10 15

tcg gcg acg acg cag aag gcg gag aag gag gtc acg cgc gtc 90
 Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Val
 20 25 30

<210> 40
<211> 30
<212> PRT
<213> Artificial Sequence

<400> 40
Thr Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln Glu
1 5 10 15
Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Val
20 25 30

<210> 41
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)..(93)

<220>
<223> Description of Artificial Sequence:chimeric loop 3
sequence

<400> 41
acc ggc cag ctc gtc ttc acg gtc aag gag gcg gcg gcg cag cag cag 48
Thr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln
1 5 10 15
gag tcg gcg acg acg cag aag gcg gag aag gag gtc acg cgc gtc 93
Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Val
20 25 30

<210> 42
<211> 31
<212> PRT
<213> Artificial Sequence

<400> 42
Thr Gly Gln Leu Val Phe Thr Val Lys Glu Ala Ala Ala Gln Gln Gln
1 5 10 15
Glu Ser Ala Thr Thr Gln Lys Ala Glu Lys Glu Val Thr Arg Val
20 25 30

<210> 43
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: high affinity
analog

<400> 43
Val Leu Glu Asp Leu Lys Ser Cys Gly Leu Phe Gly
1 5 10

<210> 44
<211> 11
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:random peptide

<400> 44
Ser Ser Val Phe Leu Val Val Asp Arg Ser Arg
1 5 10

<210> 45
<211> 91
<212> DNA
<213> Halobacterium salinarium

<400> 45
cctgcagggt cgctggactc atccacctca gcattcaccc tgctctttgg tgtgctactc 60
gttctatgac accctcggac caatactggc t 91

<210> 46
<211> 266
<212> DNA
<213> human

<220>
<221> CDS
<222> (2)..(265)

<400> 46
g tac atc ctg tac gtg ctg ttc ttc ggg ttc acc cgc gtc ttc cag gag 49
Tyr Ile Leu Tyr Val Leu Phe Phe Gly Phe Thr Arg Val Phe Gln Glu
1 5 10 15

gcg aag cgc cag ctc cag aag atc gac aag tcc gag ggc cgc ttc cac 97
Ala Lys Arg Gln Leu Gln Lys Ile Asp Lys Ser Glu Gly Arg Phe His
20 25 30

gtc cag aac ctc tcc cag gtc gag cag gac ggc cgc acc ggc cac ggc 145
Val Gln Asn Leu Ser Gln Val Glu Gln Asp Gly Arg Thr Gly His Gly
35 40 45

ctc cgc cgc tcc tcc aag ttc tgc ctc aag gag cac aag gcg ctc aag 193
Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys Ala Leu Lys
50 55 60

acc ctc gag gtc gca tcc acg ttc aaa gta ctg cgt aac gtt acc gtt 241
Thr Leu Glu Val Ala Ser Thr Phe Lys Val Leu Arg Asn Val Thr Val
65 70 75 80

gtg ttg tgg tcc gcg tat ccc tcg t 266
Val Leu Trp Ser Ala Tyr Pro Ser
85

<210> 47
<211> 88
<212> PRT
<213> human

<400> 47
Tyr Ile Leu Tyr Val Leu Phe Phe Gly Phe Thr Arg Val Phe Gln Glu
1 5 10 15
Ala Lys Arg Gln Leu Gln Lys Ile Asp Lys Ser Glu Gly Arg Phe His
20 25 30
Val Gln Asn Leu Ser Gln Val Glu Gln Asp Gly Arg Thr Gly His Gly
35 40 45
Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys Ala Leu Lys
50 55 60
Thr Leu Glu Val Ala Ser Thr Phe Lys Val Leu Arg Asn Val Thr Val
65 70 75 80
Val Leu Trp Ser Ala Tyr Pro Ser
85

<210> 48
<211> 90
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 48
ggatgcgacc tcgaggggtct tgagcgcctt gtgctccttg aggcagaact tggaggagcg 60
gcggaggcgc tggccggtgc ggccgtcctg 90

<210> 49
<211> 89
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
primer

<400> 49
ggatacgcgg accacaacac aacggtaacg ttacgcagta ctttgaacgt ggatgcgacc 60
tcgaggggtct tgagcgcctt gtgctcctt 89

<210> 50
<211> 24
<212> DNA
<213> Artificial Sequence

1911. 1912. 1913. 1914. 1915. 1916. 1917. 1918. 1919. 1920. 1921. 1922. 1923. 1924. 1925. 1926. 1927. 1928. 1929. 1930. 1931. 1932. 1933. 1934. 1935. 1936. 1937. 1938. 1939. 1940. 1941. 1942. 1943. 1944. 1945. 1946. 1947. 1948. 1949. 1950. 1951. 1952. 1953. 1954. 1955. 1956. 1957. 1958. 1959. 1960. 1961. 1962. 1963. 1964. 1965. 1966. 1967. 1968. 1969. 1970. 1971. 1972. 1973. 1974. 1975. 1976. 1977. 1978. 1979. 1980. 1981. 1982. 1983. 1984. 1985. 1986. 1987. 1988. 1989. 1990. 1991. 1992. 1993. 1994. 1995. 1996. 1997. 1998. 1999. 2000. 2001. 2002. 2003. 2004. 2005. 2006. 2007. 2008. 2009. 2010. 2011. 2012. 2013. 2014. 2015. 2016. 2017. 2018. 2019. 2020. 2021. 2022. 2023. 2024. 2025. 2026. 2027. 2028. 2029. 2030. 2031. 2032. 2033. 2034. 2035. 2036. 2037. 2038. 2039. 2040. 2041. 2042. 2043. 2044. 2045. 2046. 2047. 2048. 2049. 2050. 2051. 2052. 2053. 2054. 2055. 2056. 2057. 2058. 2059. 2060. 2061. 2062. 2063. 2064. 2065. 2066. 2067. 2068. 2069. 2070. 2071. 2072. 2073. 2074. 2075. 2076. 2077. 2078. 2079. 2080. 2081. 2082. 2083. 2084. 2085. 2086. 2087. 2088. 2089. 2090. 2091. 2092. 2093. 2094. 2095. 2096. 2097. 2098. 2099. 2100. 2101. 2102. 2103. 2104. 2105. 2106. 2107. 2108. 2109. 2110. 2111. 2112. 2113. 2114. 2115. 2116. 2117. 2118. 2119. 2120. 2121. 2122. 2123. 2124. 2125. 2126. 2127. 2128. 2129. 2130. 2131. 2132. 2133. 2134. 2135. 2136. 2137. 2138. 2139. 2140. 2141. 2142. 2143. 2144. 2145. 2146. 2147. 2148. 2149. 2150. 2151. 2152. 2153. 2154. 2155. 2156. 2157. 2158. 2159. 2160. 2161. 2162. 2163. 2164. 2165. 2166. 2167. 2168. 2169. 2170. 2171. 2172. 2173. 2174. 2175. 2176. 2177. 2178. 2179. 2180. 2181. 2182. 2183. 2184. 2185. 2186. 2187. 2188. 2189. 2190. 2191. 2192. 2193. 2194. 2195. 2196. 2197. 2198. 2199. 2200. 2201. 2202. 2203. 2204. 2205. 2206. 2207. 2208. 2209. 2210. 2211. 2212. 2213. 2214. 2215. 2216. 2217. 2218. 2219. 2220. 2221. 2222. 2223. 2224. 2225. 2226. 2227. 2228. 2229. 2230. 2231. 2232. 2233. 2234. 2235. 2236. 2237. 2238. 2239. 2240. 2241. 2242. 2243. 2244. 2245. 2246. 2247. 2248. 2249. 2250. 2251. 2252. 2253. 2254. 2255. 2256. 2257. 2258. 2259. 2260. 2261. 2262. 2263. 2264. 2265. 2266. 2267. 2268. 2269. 2270. 2271. 2272. 2273. 2274. 2275. 2276. 2277. 2278. 2279. 2280. 2281. 2282. 2283. 2284. 2285. 2286. 2287. 2288. 2289. 2290. 2291. 2292. 2293. 2294. 2295. 2296. 2297. 2298. 2299. 2300. 2301. 2302. 2303. 2304. 2305. 2306. 2307. 2308. 2309. 2310. 2311. 2312. 2313. 2314. 2315. 2316. 2317. 2318. 2319. 2320. 2321. 2322. 2323. 2324. 2325. 2326. 2327. 2328. 2329. 2330. 2331. 2332. 2333. 2334. 2335. 2336. 2337. 2338. 2339. 2340. 2341. 2342. 2343. 2344. 2345. 2346. 2347. 2348. 2349. 2350. 2351. 2352. 2353. 2354. 2355. 2356. 2357. 2358. 2359. 2360. 2361. 2362. 2363. 2364. 2365. 2366. 2367. 2368. 2369. 2370. 2371. 2372. 2373. 2374. 2375. 2376. 2377. 2378. 2379. 2380. 2381. 2382. 2383. 2384. 2385. 2386. 2387. 2388. 2389. 2390. 2391. 2392. 2393. 2394. 2395. 2396. 2397. 2398. 2399. 2400. 2401. 2402. 2403. 2404. 2405. 2406. 2407. 2408. 2409. 2410. 2411. 2412. 2413. 2414. 2415. 2416. 2417. 2418. 2419. 2420. 2421. 2422. 2423. 2424. 2425. 2426. 2427. 2428. 2429. 2430. 2431. 2432. 2433. 2434. 2435. 2436. 2437. 2438. 2439. 2440. 2441. 2442. 2443. 2444. 2445. 2446. 2447. 2448. 2449. 2450. 2451. 2452. 2453. 2454. 2455. 2456. 2457. 2458. 2459. 2460. 2461. 2462. 2463. 2464. 2465. 2466. 2467. 2468. 2469. 2470. 2471. 2472. 2473. 2474. 2475. 2476. 2477. 2478. 2479. 2480. 2481. 2482. 2483. 2484. 2485. 2486. 2487. 2488. 2489. 2490. 2491. 2492. 2493. 2494. 2495. 2496. 2497. 2498. 2499. 2500. 2501. 2502. 2503. 2504. 2505. 2506. 2507. 2508. 2509. 2510. 2511. 2512. 2513. 2514. 2515. 2516. 2517. 2518. 2519. 2520. 2521. 2522. 2523. 2524. 2525. 2526. 2527. 2528. 2529. 2530. 2531. 2532. 2533. 2534. 2535. 2536. 2537. 2538. 2539. 2540. 2541. 2542. 2543. 2544. 2545. 2546. 2547. 2548. 2549. 2550. 2551. 2552. 2553. 2554. 2555. 2556. 2557. 2558. 2559. 2560. 2561. 2562. 2563. 2564. 2565. 2566. 2567. 2568. 2569. 2570. 2571. 2572. 2573. 2574. 2575. 2576. 2577. 2578. 2579. 2580. 2581. 2582. 2583. 2584. 2585. 2586. 2587. 2588. 2589. 2590. 2591. 2592.

<400> 50
tgtacatgta catcctgtac gtgc 24

```
<210> 51
<211> 90
<212> DNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:oligonucleotide
        primer
```

```
<400> 51
atcctgtacg tgctgttctt cgggttcacc cgcgtcttcc aggaggcgaa gcgccagctc 60
cagaagatcg acaagtccga gggccgcttc                                     90
```

```
<210> 52
<211> 90
<212> DNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer
```

```
<400> 52
aagcgccagc tccagaagat cgacaagtcc gagggccgct tccacgtcca gaacctctcc 60
caagtcgaqc aggacggccg caccggccac                                     90
```

```
<210> 53
<211> 35
<212> DNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:oligonucleotide
        primer
```

<400> 53
gctgccgata agccacacga ctggatacgc ggacc 35